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ANTI-HERPESVIRAL AGENT

The present invention relates to an anti-viral agent effective against herpesviruses and to an assay for screening for other suitable anti-viral agents.

Herpesviruses include Herpes Simplex Virus types 1 and 2 (HSV-1 and HSV-2), Human Cytomegalovirus (HCMV), Epstein-Barr Virus (EBV) and Equine herpesviruses 1 and 4 (EHV-1 and EHV-4). The term "Herpesvirus" is used herein to refer to any virus of the herpesvirus family, including viruses in the α group (e.g. HSV 1 & 2, EHV 1 & 4), the β group (e.g. HCMV) and in the γ group (e.g. EBV).

Infections due to HSV have been successfully treated for many years through use of the drug acyclovir, a nucleoside analogue. Acyclovir is relatively non-toxic to the human host since it does not adversely affect the activity of the mammalian homologue of the targeted viral protein. However, similar low toxicity regimes for treating all herpesviruses have not yet been found. Whilst HCMV is treatable via use of the drug gancyclovir (Coen, 1992) the application of this drug is limited by its toxicity, poor bioavailability and

1 the emergence of drug-resistant variants (reviewed by
2 Coen 1992; Haffey & Field 1995; Filley et al 1995). A
3 low-toxicity treatment for HCMV is particularly of
4 interest as infection by this virus can cause
5 congenital abnormalities in the newborn exposed to the
6 virus by maternal transmission, and is also extremely
7 problematic to immunocompromised patients, for example
8 patients suffering from AIDS, or those on
9 immunosuppressive therapy for cancer or following organ
10 transplant.

11
12 The genome of herpes simplex virus type 1 (HSV-1)
13 encodes seven proteins essential for origin dependent
14 viral DNA synthesis (Wu et al., 1988) The genes
15 encoding these proteins, and their protein products,
16 are known in the art as UL5, UL8, UL9, UL29, UL30, UL42
17 and UL52. (McGeoch et al., 1988). Frequently the names
18 of the genes are italicized, eg *UL5*, to avoid possible
19 ambiguities. The UL30 protein, the catalytic subunit
20 of the heterodimeric HSV-1 DNA polymerase, is also
21 known as POL. Homologues of all seven genes have been
22 identified in other alphaherpesviruses and human
23 herpesviruses 6 and 7 (HHV-6 and HHV-7). Other beta-
24 and gammaherpesviruses encode homologues of all these
25 proteins except UL9. For convenience the terminology
26 of the HSV-1 proteins will be used to refer not only to
27 that particular protein but also its equivalent in
28 other herpesviruses. Thus, as used herein the term
29 "UL8" refers not only to UL8 of HSV-1 itself, but also
30 to the HCMV homologue UL102 and to equivalent homologues
31 in other herpesviruses. Similarly, as used herein the
32 term "POL" (or "UL30") refers not only to POL of HSV-1
33 itself, but also to the HCMV homologue UL54 and to
34 equivalent homologues in other herpesviruses.

35
36 The functions of these proteins and their interactions

may be summarised as follows. The UL9 product is an origin-binding protein (OBP) and the UL29 product (ICP8) a single-stranded DNA binding protein. These two proteins can interact via the C-terminus of UL9 (Boehmer and Lehman, 1993; Boehmer *et al.*, 1994). The UL30 protein (POL) and UL42 proteins comprise the catalytic and accessory components, respectively, of a dimeric DNA polymerase (reviewed by Challberg, 1991; Weller, 1991) and interact via residues at or near the C-terminus of POL (Digard & Coen, 1990; Digard *et al.*, 1993, 1995; Marsden *et al.*, 1994; Stow *et al.*, 1993; Tenney *et al.*, 1993). The UL5, UL8 and UL52 proteins form a trimeric complex that exhibits both DNA helicase and DNA primase activities (Dodson *et al.*, 1989; Crute *et al.*, 1989). The UL5 protein is largely responsible for DNA helicase activity (Gorbalenya *et al.*, 1989; Zhu & Weller, 1992), and the UL52 protein contributes an essential role in DNA priming (Klinedinst & Challberg, 1994; Dracheva *et al.*, 1995) and these two proteins can form a stable subassembly that retains both functions (Calder & Stow, 1990; Dodson & Lehman, 1991; Crute *et al.*, 1991). The UL8 component has auxiliary effects on the DNA primase activity, stimulating primer synthesis and/or utilization on a natural-sequence single-stranded DNA template (Sherman *et al.*, 1992; Tenney *et al.*, 1994), and is also required for efficient nuclear entry of the trimeric complex. (Calder *et al.*, 1992; Marsden *et al.*, 1996). UL8 is capable of binding separately to the UL5 and UL52 proteins and can also interact specifically with UL9 (McLean *et al.*, 1994). The latter interaction with OBP may serve to recruit the helicase-primase into an initiation complex at the viral origins.

Further evidence for the occurrence of multiple interactions between DNA replication proteins has been

provided by immunofluorescence experiments. In cells infected with HSV-1 in the presence of inhibitors of viral DNA synthesis UL29 (ICP8) localises to punctate structures within the nucleus termed "pre-replicative sites" (Quinlan *et al.*, 1984). The requirement for each of the DNA replication proteins in the formation of these sites has been studied by the use of viral mutants with defects in individual replication proteins (Liptak *et al.*, 1996; Lukonis *et al.*, 1996). It was observed that proteins UL5, UL8, UL9 and UL52 are all necessary for the localisation of UL29 (ICP8) into pre-replicative sites and that mutants with defects in any of the other six DNA replication genes are affected in the ability of POL to localize to these sites. Although these data suggest that the DNA polymerase holoenzyme is the last component to be recruited (Liptak *et al.*, 1996) they do not identify the specific interactions involved in this event.

It has now been found that the protein UL8 interacts with POL. Further, it has been found that disruption of the POL/UL8 interaction is possible. Examples of molecules, monoclonal antibodies and peptides that specifically disrupt the interaction have been identified.

The present invention provides an anti-viral agent capable of combatting replication of a herpesvirus by interfering with the association of UL8 and POL (as defined above).

Both the UL8 and POL proteins of HSV-1 have been previously described in the literature (e.g. Parry *et al.*, 1993; Gottlieb *et al.*, 1990).

Furthermore the amino acid/DNA sequences of UL8 and POL

1 from HSV-1 are available from publically accessible
2 Genbank and EMBL databases under Nos. P10192/M19120 and
3 P04293/M12356 (and several other entries),
4 respectively.

5
6 The UL8/POL association is an association between two
7 viral proteins, that are significantly different from
8 any protein in the mammalian host organism (for HSV-1,
9 the host is humans). Although homologues of POL are
10 present in mammalian cells they are considerably
11 diverged. No cellular homologue of UL8 is known. For
12 the virus to overcome disruption of such a viral
13 protein: viral protein interaction a double mutation,
14 i.e. a mutation in each of the viral proteins involved,
15 may be required. Alternatively the range of single
16 mutations that overcome disruption, yet allow the two
17 proteins to interact normally may be severely
18 restricted. The probability of such reversion
19 occurring is thus relatively low rendering this type of
20 interaction attractive as a potential target for
21 therapeutic agents. Additionally, as neither UL8 nor
22 POL have close homologues in mammalian cell metabolism,
23 the toxicity of an agent which specifically interacts
24 with these proteins will be low.

25
26 The anti-viral agent may be a peptide or more
27 preferably a non-peptidal compound having
28 peptidomimetic properties. Such a non-peptidal
29 compound will be preferred since it will be resistant
30 to enzymic breakdown by peptidases. Suitable anti-
31 viral compounds may include peptides having an amino
32 acid sequence derived from the C-terminal or C-proximal
33 region of UL8, a functional equivalent of such a
34 peptide, or a peptidomimetic compound therefor.

35
36 The computer program "Predict-Protein" (EMBL-

Heidelberg) makes a strong prediction of the presence of an alpha-helical region near the C-terminus of HSV-1 UL8 (amino acids 709-728) with the very C-terminus (residues 729-750) predicted to be in looped or extended structures (perhaps as a "tail"). The secondary structure predictions for the C-terminal regions of the UL8 homologues of bovine herpesvirus 1 (BHV-1), human cytomegalovirus (HCMV, betaherpesvirus) and Epstein-Barr virus (EBV, gammaherpesvirus) are all similar in that an alpha-helical region of approximately 20 amino acids is strongly predicted to occur within 10-26 amino acids of the C-terminus. The most inhibitory HSV-1 peptide we have identified (peptide 7, amino acids 719-738) is derived from across the junction of the predicted alpha-helix and "tail" portions at the C-terminus of UL8 and is 20 amino acids in length. We consider it likely that the predicted conserved structures in the C-terminal regions of the other herpesvirus UL8 homologues discussed above are similarly involved in interactions with the POL homologues and peptides representing similar regions might be able to disrupt the POL/UL8 interactions in these viruses. Thus the peptide is preferably derived from the free "tail" portion and/or the α -helix portion of the C-terminus of UL8. Optionally the peptide is as small as possible, eg less than 6 amino acids, but can be eg 10, 14 or more amino acids in length, particularly where the peptide is derived wholly or partially from the α -helical region of the C-terminus of UL8.

Suitable peptides are set out in Table 2. In the table of inhibitory peptides the lower the IC_{50} value the greater the inhibitory activity. Peptides Nos 5 and 7 are especially effective as anti-viral agents. Peptide 7 corresponding to α 719-738 was the most inhibitory and is preferred. Functional analogues of the peptides

1 of Table 2 (especially Nos 5 and 7) and peptidomimetic
2 compounds therefor are likewise suitable anti-viral
3 agents. Peptides derived from α s 722-738 are
4 particularly suitable.

5

6 The anti-viral agent is preferably effective against a
7 herpesvirus selected from HSV, HCMV, Human herpesvirus
8 8 (HHV8), EBV and EHV 1 & 4. HCMV is of particular
9 interest. The antiviral agent is preferably also
10 effective against proteins homologous to UL8 and POL
11 (eg UL102 and UL54 respectively for HCMV). Generally
12 the anti-viral agent will be selected to mimic at least
13 a portion at or near the C-terminus of the UL8
14 homologue of the specific target virus.

15

16 In a further aspect, the present invention provides an
17 assay to determine the ability of a test substance to
18 interfere with the association of UL8 and POL. The
19 assay comprises the following steps:

20

21 i) providing a first viral component;

22

23 ii) exposing said first viral component to a test
24 substance followed by a second viral component, or
25 exposing said first viral component to a second
26 viral component followed by a test substance;

27

28 iii) washing to remove any second viral component
29 and/or test substance not associated with the
30 first viral component; and

31

32 iv) detecting the presence, and optionally determining
33 the amount, of second viral component associated
34 with said first viral component.

35

36 The first or second viral components may be localised

1 on a surface, such as a blotting membrane, or an assay
2 plate for ELISA etc. Preferably the first component is
3 immobilised in such a manner, although the invention
4 contemplates the possibility of the assay being carried
5 out in solution.

6
7 The first viral component may be POL or UL8. Where the
8 first viral component is POL, the second viral
9 component will be UL8. Where the first viral component
10 is UL8, the second viral component will be POL.

11
12 If the assay is to test the ability of the test
13 substance to interfere with UL54/UL102 association, the
14 first viral component may be UL54 or UL102. Where the
15 first viral component is UL54, the second viral
16 component will be UL102. Where the first viral
17 component is UL102, the second viral component will be
18 UL54.

19
20 Detection of the presence and/or amount of second viral
21 component associated with the first viral component may
22 be conducted by any convenient means. Generally
23 detection may be via a monoclonal antibody, the
24 presence of which is established by exposure to a
25 second labelled monoclonal antibody in a typical ELISA-
26 style assay. Alternatively, the second viral component
27 may be labelled (eg radioactively) to determine its
28 binding to the first viral component.

29
30 Suitable monoclonal antibodies (Mabs) for use in the
31 assay of the present invention have been produced (see
32 Examples 2 and 3) and form a further aspect of the
33 present invention. In particular the POL-specific Mab
34 13185 is suitable for use in the assay of the present
35 invention where POL of HSV-1 is the second viral
36 component. Mabs 804 and 805 are UL8-specific Mabs and

1 are suitable for use in the present invention where UL8
2 of HSV-1 is the second viral component. Hybridoma
3 cell-lines have been deposited for Mabs 13185 and 805
4 at the European collection of animal cell cultures at
5 ECACC, Porton Down, Wiltshire on 26 July 1996 under
6 Accession Nos 96072640 and 96072639 respectively.

7
8 Identification of MAb 814 as an antibody that inhibits
9 the POL/UL8 interaction and the mapping of its epitope
10 to between amino acids 470 and 671 suggests that the
11 C-terminus may not be the only region of UL8 to
12 contribute to POL binding. For the POL/UL42
13 interaction the C-terminus was found to contribute 75%
14 of the binding energy (Marsden et al 1994). The
15 relative contribution of different regions of UL8 to
16 POL binding remains to be determined.

17
18 By analogy with other DNA replication systems it is
19 considered likely that initiation of HSV-1 DNA
20 synthesis involves the formation of an initiation
21 complex at one or more of the replication origins. The
22 first stage in this process would be the binding of UL9
23 to its recognition sequence. The interaction of UL9
24 with UL8 might then serve to recruit the viral
25 helicase-primase complex (UL5, UL8 and UL52) (McLean et
26 al., 1994). In addition, ICP8 both interacts
27 physically with UL9 and can stimulate its helicase
28 activity (Boehmer & Lehman, 1993; Boehmer et al.,
29 1994). These five proteins together therefore have the
30 potential to open up the duplex DNA in the origin
31 region and synthesize RNA primers. The interaction
32 between POL and UL8 which we have now identified may
33 play an important role in bringing the viral DNA
34 polymerase (POL/UL42 heterodimer) into the complex to
35 initiate DNA synthesis. In addition a direct physical
36 interaction between the polymerase and helicase-primase

1 complexes may be important in co-ordinating the
2 unwinding of the duplex and the synthesis of RNA
3 primers on the lagging strand at the advancing
4 replication fork. This model, summarized in Figure 10,
5 is entirely compatible with that proposed by Liptak et
6 al. (1996) in which UL5, UL8, UL9, UL29(ICP8) and UL52
7 are assembled at prereplicative sites followed by
8 recruitment of POL, which is facilitated by UL42. Our
9 finding provides the basis for the recruitment of the
10 POL/UL42 complex. Amongst the many questions that
11 remain to be answered is whether the affinities of the
12 different proteins for each other is influenced by the
13 presence of other proteins in the complex. It is
14 possible, for example, that binding of POL to UL8
15 reduces the affinity of UL8 for UL9 allowing the
16 helicase-primase-polymerase complex to migrate away
17 from the origin to the replication forks.

18
19 The interaction of POL with UL8 may represent a
20 possible new target for the action of an antiviral
21 agent. A UL8 protein lacking the C-terminal 34 amino
22 acids is unable to support viral DNA synthesis in a
23 transient transfection assay indicating that this
24 region of the UL8 protein performs an essential
25 replicative function. Although this provides evidence
26 consistent with a key role for the UL8/POL interaction,
27 it should be noted that we cannot exclude the
28 possibility that this region of the protein is also
29 necessary for some other essential function.

30
31 Our identification of peptides that block this
32 interaction should also encourage further studies of
33 this region and the search for more potent inhibitors.
34 In the case of the HSV ribonucleotide reductase,
35 following the initial discovery that peptides
36 corresponding to the C-terminus of the small subunit

1 inhibited enzyme activity (Cohen et al., 1986; Dutia et
2 al., 1986), it proved possible to identify more active
3 peptidomimetic compounds that could function
4 intracellularly (Luizzi et al., 1994; Moss et al.,
5 1995). The POL/UL8 interaction may be an especially
6 attractive new target for two reasons. First, both
7 proteins are present in infected cells in low amount in
8 contrast to POL/UL42 and R1/R2 where one or both of the
9 interacting proteins are abundant viral products.
10 Second, the POL/UL8 interaction appears to be
11 relatively weak as suggested by the observation that in
12 contrast to POL/UL42 and R1/R2 they do not co-purify
13 from infected cells and also by the ability of peptide
14 7 to block the interaction equally effectively when
15 pre-incubation with POL was omitted. Such a weak
16 interaction may be more readily blocked by an antiviral
17 compound than a strong interaction.

18
19 Mabs 817, 818 and 819 all recognised peptide 5, that
20 corresponds to residues 722 to 750 of UL8, and to a
21 lesser extent peptide 3 (amino acids 726-750). However
22 the Mabs do not recognise peptide 2 (amino acids 728-
23 750) or peptide 7 (amino acids 719-738). It is
24 therefore probable that all three MABs recognize the
25 same epitope located within the C-terminal 29 amino
26 acids of UL8 and minimally involving the region
27 spanning amino acids 727-739.

28
29 The present invention also provides a method of
30 combatting replication of a herpesvirus, said method
31 comprising providing an agent capable of disrupting the
32 association UL8 and POL.

33
34 Further, the present invention provides a method of
35 combatting an infection caused by a herpesvirus, said
36 method comprising administering an antiviral agent as

described above.

Additionally the present invention provides the use of an agent capable of interfering with association of POL/UL8 for combatting herpesvirus replication or infection.

FIGURE LEGENDS

Figure 1. Co-precipitation of POL and UL8 protein by the UL8-specific MAb804. Lanes 1 to 3 show [³⁵S]-methionine-labelled extracts from Sf cells infected with AcUL8 (lane 1), AcUL30 (lane 2) or doubly with AcUL8 and AcUL30 (lane 3). The proteins precipitated from these extracts are shown in lanes 4 to 6 respectively. Proteins were separated on 8.5% SDS-polyacrylamide gels and were visualized by autoradiography. The positions of the POL and UL8 proteins are indicated.

Figure 2. POL/UL8 interaction ELISAs. Panel A. UL8 protein was added to microtiter wells pre-coated with 0.04µg POL (●,■) or uncoated (○,□). Bound UL8 protein was detected with MAb804 (□,■) or MAb805 (○,●) which in turn were detected with an HRP-conjugated anti-mouse MAb and colorimetric substrate. Panel B. POL was added to microtiter wells pre-coated with 0.4µg UL8 protein (■) or uncoated (□). Bound POL was detected with MAb 13185 which in turn was detected with an HRP-conjugated anti-mouse MAb and colorimetric substrate.

Figure 3. Inhibition of the POL/UL8 interaction by UL8-specific MAbs. Ascitic fluid from UL8-specific MAbs and two control MAbs (RwP3 and 105gD) were serially two-fold diluted and incubated for 1h at 37°C with 0.2µg UL8 protein. The mixture was then added to

1 microtiter wells coated with 0.04 μ g POL. After 1h the
2 plates were washed and bound UL8 protein was detected
3 with MAB 804 or MAB 805 as described. The absorbance
4 in each well was expressed relative to that (0.945 OD
5 units) observed in the absence of antibody. The
6 dilutions of ascitic fluid were as follows: ■, 8-fold;
7 □, 16-fold; ▤, 32-fold; ≡, 64-fold; ▥, 128-fold;
8 ≡, 256-fold; ▧, 512-fold and □, 1024-fold. The
9 designation of each MAb is shown below the absorbance
10 values obtained for that MAb.

11
12 *Figure 4.* Approximate mapping of the epitopes
13 recognized by the UL8-specific MABs. Four replicate
14 SDS-PAGE gels were loaded with total proteins from BHK
15 cells lipofected with the plasmids indicated.
16 Following electrophoresis and electroblotting of the
17 proteins onto nitrocellulose sheets the blots were
18 incubated with 1:2000 dilutions of polyclonal anti-UL8
19 antiserum (a), MAB 0811 (b), MAB 0814 (c) or MAB 0817
20 (d). The blots were washed and incubated with 1:7500
21 dilutions of anti-rabbit (a) or anti-mouse (b-d) IgG
22 alkaline phosphatase conjugated antibody and developed
23 as described in Methods. The sizes of the prestained
24 molecular weight markers (Sigma) are indicated.

25
26 *Figure 5.* Fine mapping of the epitopes recognized by
27 MABs 817, 818 and 819. The reactivity of the MABs with
28 peptides 1 - 8 (Table 2) was tested by ELISA. The bars
29 show the absorbance observed with a 100-fold dilution
30 of each MAB. The MABs are denoted by the different
31 cross-hatching as follows: ▤, MAB 817; ≡, MAB
32 818; ≡, MAB 819.

33
34 *Figure 6.* Inhibition of the POL/UL8 interaction by UL8
35 peptides. Different concentrations of peptides 5 (O)
36 and 7 (◇) and the control peptide RT85 (□) (Table 2)

1 were incubated for 15 min at room temperature with
2 0.15µg POL and then added to microtiter wells coated
3 with 0.2µg UL8 protein. After 1h the plates were
4 washed and bound POL was detected with MAb13185.

5
6 *Figure 7.* The sequence of amino acids in peptide 7 is
7 important for inhibition of the POL/UL8 interaction.
8 Different concentrations of peptide 7 (□) and peptide
9 7J (◇), a jumbled version of peptide 7, (Table 2) were
10 incubated for 15 min at room temperature with 0.15µg
11 POL and then added to microtiter wells coated with
12 0.2µg UL8 protein. After 1h the plates were washed and
13 bound POL was detected with MAb13185.

14
15 *Figure 8.* Inhibition of the POL/UL8 interaction does
16 not require prior incubation of peptide 7 with POL.
17 Different concentrations of peptide 7 and POL (0.15µg)
18 were added to microtiter wells coated with 0.2µg UL8
19 protein: the peptide was added either a few seconds
20 after POL (□), or was first pre-incubated with POL for
21 15 min at room temperature (◇). After 1h the plates
22 were washed and bound POL was detected with MAb13185.

23
24 *Figure 9.* Alignment of the sequences of peptides 7J, 7
25 and 5. The * indicates the positions at which amino
26 acids in the three peptides are identical.

27
28 *Figure 10.* Interactions between the HSV-1 DNA
29 replication proteins. The thin, medium and thick
30 arrows indicate relative strengths of interaction. In
31 addition to binding to UL29 and UL8, UL9 also binds
32 specifically to the viral replication origins. The
33 UL9/UL29, UL9/UL8 and UL8/POL interactions are likely
34 to be important in assembling an initiation complex at
35 the origins following the initial binding of UL9.

36

Figure 11. Epitope mapping of POL-specific MAb's by western blotting. Extracts of *E. coli* cells expressing fragments 1-7 of gene UL30 encoding residues 1-212, 162-316, 308-658, 597-975, 875-1119, 1072-1145 and 1128-1235 respectively, were separated by SDS-PAGE (lanes 1-7). Lane 8 contains an extract from *E. coli* cells transformed with the vector pQE32 to serve as a control. Four such gels were blotted onto nitrocellulose membranes and probed with polyclonal antiserum 514 (panel A), MAb 13088 (panel B), MAb13129 (panel C) and MAb 13185 (panel D). The positions to which molecular weight markers (M_r s 46,000, 30,000, 21,500, 14,300, 6,500, and 3,400) migrate, are shown by arrows on the left of each panel.

Figure 12. Coomassie-blue stained gel of purified proteins UL102 (lane 2) and UL54 (lane 3). Proteins were separated on an SDS-10% polyacrylamide gel. The numbers to the left of the gel show the molecular weights ($\times 10^{-3}$) of the marker proteins that were electrophoresed in lane 1.

Figure 13. Reactivity of antiserum 373, made against a peptide corresponding to amino acids 809-823 of the predicted UL102 ORF, with extracts of Sf cells infected with recombinant virus AcUL102 and proteins at different stages of purification. Proteins were separated on an SDS-10% polyacrylamide gel, transferred to a nitrocellulose membrane and reacted with antiserum 373 (lanes 1 -5) or the pre-immune serum (lanes 6 - 10). The electrophoresed proteins were two different preparations of AcUL102-infected Sf cells (EXT-1 and EXT-2, lanes 2, 3, 7 and 8) together with peak UL102-containing fractions from the DEAE column (DEAE-2, lanes 1 and 6) and hydroxylapatite column (HA-2, lanes 4 and 9). Lanes 5 and 10 contain

1 molecular weight markers.

2

3 *Figure 14.* Specificity of antiserum 144 for UL54.

4 ELISA wells were coated with 0.04 mg (●,○) or 0 µg
5 (▼,▽) of UL54 protein and reacted with antiserum 144
6 (●,▼) or the pre-immune serum (○,▽) as described
7 previously (Marsden et al., 1994). The sera were
8 initially diluted 5-fold followed by serial 2-fold
9 dilutions. Bound antibody was detected with
10 HRP-conjugated protein A and colorimetric substrate.

11

12 *Figure 15.* DNA-dependent DNA polymerase activity of
13 purified UL54 protein. Incorporation of [³H]dTTP into
14 an poly(dA)-oligo(dT)₁₂₋₁₈ template by 10 ng protein (●)
15 or no protein (■).

16

17 *Figure 16.* The HSV-1 UL30/POL interaction can be
18 detected by rabbit antibody 113 that was raised against
19 the C-terminal 15 amino acids of HSV-1 UL30. The data
20 is presented as 6 groups (A - F) each comprising 4
21 absorbance values (1 - 4). The absorbance values
22 represent data from the interaction assay as follows:
23 1, both UL30 and UL8 proteins present; 2, UL8 only; 3,
24 UL30 only; 4, both UL30 and UL8 proteins absent. The
25 groups correspond to absorbance values produced with
26 detecting antibodies as follows: A, MAb 13815 diluted
27 1/50; B, no monoclonal antibody; C - E, Rabbit
28 antiserum 113 diluted 1/10³, 1/10⁴, 1/10⁵ respectively;
29 F no rabbit antiserum. The presence of bound antibody
30 was detected with HRP-conjugated goat anti-mouse
31 antibody (groups A and B) or HRP-conjugated protein A
32 (groups C - E) and colorimetric substrate.

33

34 *Figure 17.* UL54/UL102 interaction ELISA. UL54
35 protein was added to microtiter wells pre-coated with
36 UL102 protein. The amounts of UL102 protein used to

1 coat the wells were as follows: 0.4 μ g (●), 0.2 μ g
2 (○), 0.1 μ g (▼), 0.02 μ g (▽), or uncoated (■). Bound
3 UL54 was detected with rabbit antiserum 114 which in
4 turn was detected with HRP-conjugated protein A and
5 colorimetric substrate.

6
7 *Figure 18.* Inhibition of the UL54/UL102 interaction
8 by UL102 peptides. Different concentrations of
9 peptides 1 (●) and 2 (○) and the control peptide RT85
10 (▼) (Table 4) were added with 0.4 μ g UL54 to microtiter
11 wells coated with 0.4 μ g UL102 protein. After 1h the
12 plates were washed and bound UL54 was detected with
13 HRP-conjugated protein A and colorimetric substrate.

14
15 **SEQUENCE LISTINGS**

16
17 SEQ ID NO 1 : Peptide 1 (Table 2)
18 SEQ ID NO 2 : Peptide 2 (Table 2)
19 SEQ ID NO 3 : Peptide 3 (Table 2)
20 SEQ ID NO 4 : Peptide 4 (Table 2)
21 SEQ ID NO 5 : Peptide 5 (Table 2)
22 SEQ ID NO 6 : Peptide 6 (Table 2)
23 SEQ ID NO 7 : Peptide 7 (Table 2)
24 SEQ ID NO 8 : Peptide 8 (Table 2)
25 SEQ ID NO 9 : Peptide 7J (Table 2)
26 SEQ ID NO 10 : Peptide RT85 (Table 2)
27 SEQ ID NO 11 : Peptide 1 (Table 4)
28 SEQ ID NO 12 : Peptide 2 (Table 4)
29 SEQ ID NO 13 : C-terminal 15 amino acids of UL54
30 SEQ ID NO 14 : Residues 809-823 of the 873 residue
31 UL102
32 SEQ ID NO 15 : Primer (Example 3)
33 SEQ ID NO 16 : Primer (Example 3)
34 SEQ ID NO 17 : Primer (Example 3)
35 SEQ ID NO 18 : Primer (Example 3)

1 The present invention will now be further described
2 with reference to the following, non-limiting,
3 examples.

4
5
6 **EXAMPLE 1 : POL/UL8 association**

7
8 **MATERIALS AND METHODS**

9
10 *Cells and recombinant baculoviruses.* *Spodoptera*
11 *frugiperda* (Sf) cells (strain IPLB-SF-21; Kitts et al.,
12 1990) were maintained in TC100 medium (Life
13 Technologies) containing 5% (v/v) fetal calf serum
14 (FCS), penicillin (100 units/ml) and streptomycin
15 (100µg/ml). The *Autographa californica* nuclear
16 polyhedrosis virus (AcNPV) recombinants AcUL30, AcUL8
17 (which overexpress POL and UL8 proteins respectively)
18 have been described (Stow, 1992; 1993). Preparation
19 and titration of virus stocks were carried out as
20 described (Brown and Faulkner, 1977; Matsuura et al.,
21 1987).

22
23 *Antibodies.* The isolation of one UL8-specific
24 monoclonal antibody (MAb) following immunisation of
25 mice with purified UL8 protein was described previously
26 (McLean et al., 1994). An additional 19 MAbs were
27 isolated from the same fusion and screened for
28 reactivity with UL8 protein by ELISA. Ascitic fluid
29 was prepared from cells secreting UL8-reactive
30 antibodies. Two control MAbs were also used in these
31 experiments: MAb RWP3 is secreted from the P3-X67-Ag8
32 myeloma cells (Kohler and Milstein, 1975) and MAb105gD
33 reacts with glycoprotein D of HSV-1 (A. Cross,
34 unpublished data). Polyclonal rabbit antiserum 094 was
35 prepared in a Sandy Half-Lop rabbit which was immunized
36 intramuscularly, first with approximately 25µg purified

1 UL8 protein (Parry *et al.*, 1993) in Freund's complete
2 adjuvant, followed by three boosts 10, 30 and 40 days
3 later using the same amount of antigen but in Freund's
4 incomplete adjuvant. The animal was sacrificed on day
5 50 and serum was collected.

6
7 *Immunoprecipitation, immunofluorescence and Western*
8 *blotting.* The procedures used to prepare
9 [³⁵S]-methionine labelled extracts from Sf cells
10 infected with recombinant baculoviruses have been
11 described in detail (McLean *et al.*, 1994). The
12 extracts were incubated with 1.0 µl of ascitic fluid of
13 UL8-specific MAb804, immune complexes were captured on
14 Protein A-Sepharose beads, proteins were separated on
15 8.5% SDS polyacrylamide gels and were then visualized
16 by autoradiography as described in detail previously
17 (McLean *et al.*, 1994). Immunofluorescence and Western
18 blotting were performed as described (Calder *et al.*,
19 1992; McLean *et al.*, 1994). Briefly, Sf cells infected
20 with AcUL8 were harvested 2 days after infection,
21 washed with PBS and solubilised with denaturing sample
22 buffer (Laemmli, 1970), separated by 10% SDS-PAGE, and
23 transferred to nitrocellulose membranes. The membranes
24 were incubated with MAbs diluted from 10²- to 10⁵-fold,
25 and bound antibodies were visualised using HRP coupled
26 to anti-mouse Ig (Sigma), and chromogenic substrate
27 4-chloro-1-naphthol (Bio-Rad).

28
29 *POL/UL8 interaction assays.* ELISA assays, similar to
30 that described for POL/UL42 (Marsden *et al.*, 1994) were
31 established with purified POL and UL8 proteins. POL
32 was extracted from Sf cells infected with recombinant
33 baculovirus AcUL30 and purified as described by
34 Gottlieb *et al.* (1990) with minor modifications
35 (Marsden *et al.*, 1994). UL8 protein was extracted
36 from Sf cells infected with recombinant baculovirus

AcUL8 and purified as described by Parry *et al.*, (1993) but substituting phenyl-Sepharose by hydroxylapatite chromatography (Dodson and Lehman, 1991). Both proteins were diluted in PBS to the required concentrations. For the first assay, microtiter wells were coated overnight with 0.02 µg of purified POL and blocked with 100 µl of 2% BSA in PBS for 1 h at 37°C. After blocking, the plates were washed extensively with PBS containing 0.3% Tween 20 and blotted dry. Then 50 µl of purified UL8, at the concentrations indicated in the text, were added to each well and incubated for 1 h at 37°C. Following further washes, 50 µl of UL8-specific MAb 804 or MAb 805 diluted 1:400 in PBS containing 2% FCS was reacted for 1 h at 37°C. The wells were again extensively washed and bound MAb was detected with 50 µl/well of HRP-conjugated goat anti-mouse IgG (Sigma) diluted 1:500 in PBS containing 2% FCS and chromogenic substrate ABTS. For the second assay, POL and UL8 proteins were added in the reverse order. Microtitre wells were coated overnight with 0.02 to 0.04 µg of purified UL8 protein and bound POL was detected with a POL-specific MAB 13185 (Marsden *et al.*, 1996) diluted 1:100. Other aspects of the two assays were identical. MAbs, diluted in PBS plus 2% fetal calf serum, and peptides, diluted in 100mM Tris-HCl (pH 8.0) plus 0.1% Tween 20, were added to the interaction assay as described in the text.

Oligopeptides. Peptides (Table 2) were synthesized by continuous flow Fmoc chemistry as previously described (Atherton and Sheppard, 1989; McLean *et al.*, 1991). Peptides were purified by preparative reverse-phase HPLC (Owsianka *et al.*, 1993). The Mr values of monomeric peptides were determined by matrix-assisted, laser desorption time-of-flight (MALDI-TOF) mass spectrometry and corresponded to the desired products.

1 *Expression of fragments of UL8 and mapping UL8-specific*
2 *MAbs.* Plasmid pE8 contains the *UL8* DNA replication
3 gene under the control of the human cytomegalovirus
4 major immediate early promoter in the vector pCMV10
5 (Stow et al., 1993) and served as parent for the
6 construction of plasmids expressing N- and C-terminally
7 truncated *UL8* proteins, designated pNΔx and pCΔx,
8 where x corresponds to the number of amino acids
9 deleted (E.C. Barnard and N.D. Stow, manuscript in
10 preparation). BHK cells (approximately 1.5×10^5 per
11 35 mm diameter petri dish) were transfected with 2 μg
12 wild type pE8 or deletion mutant DNA using liposomes
13 prepared as described by Rose et al. (1991). 30 h
14 post-transfection the cells were washed with PBS and
15 total cell proteins prepared by treating the monolayers
16 with 150 μl denaturing sample buffer (Laemmli, 1970).
17 Protein samples (usually 20 μl) were subjected to
18 electrophoresis through 9% polyacrylamide gels using a
19 Bio-Rad mini protein gel kit and electroblotted onto a
20 nitrocellulose membrane (Towbin et al., 1979).
21 Replicate membranes were reacted with a 1 in 2000
22 dilution of the MAb or with a 1 in 2000 dilution of
23 rabbit polyclonal antiserum 094 against whole *UL8*
24 protein, and bound antibody was detected using alkaline
25 phosphatase-conjugated anti-mouse or anti-rabbit IgG
26 secondary antibody, as appropriate, in conjunction with
27 the Promega Protoblot system.

28
29 *Fine mapping of MAbs with peptide-based ELISA assays.*

30 Peptides were diluted in 100 mM Tris-HCl (pH 8.0) plus
31 0.1% Tween 20 and coated overnight onto microtiter
32 wells at 1.0 μg/well in 50 μl. The wells were then
33 blocked and washed as described above. MAbs were
34 diluted 100-fold in PBS plus 2% FCS and 50 μl was added
35 to each well and incubated for 1 h. The antibody was
36 removed, plates were again washed and bound antibody

was determined with HRP-conjugated anti-mouse IgG and colorimetric substrate as described above.

RESULTS

Isolation and characterisation of MAbs reactive with UL8 protein. The isolation of a single MAb, (designated 0811) following immunisation of mice with purified UL8 has been described (McLean et al., 1994). From the same fusion a further 19 cell lines secreting MAbs reactive with UL8 protein were isolated. Ascitic fluid was developed for each cell line and screened for reactivity with UL8 protein in immunoprecipitation, immunofluorescence and Western blotting assays. Four of the MAbs were found to react strongly by Western blotting with protein(s) from uninfected BHK cells and were not studied further. The results obtained for the remaining 16 MAbs are summarized in Table 1.

Co-precipitation of POL with UL8. MAbs capable of immunoprecipitating UL8 protein have also been examined for their ability to co-precipitate other viral DNA replication proteins from [³⁵S]-methionine-labelled extracts of Sf cells mixedly infected with recombinant baculoviruses. We previously described the identification of an interaction between UL8 and UL9 using this approach (McLean et al., 1994). When extracts from Sf cells co-infected with AcUL8 and AcUL30 were reacted with MAb804, POL was found to co-precipitate with UL8 (Fig. 1, lane 6). Precipitation of POL was specific and dependent on the presence of UL8 protein since no protein of a similar size was detected in the immunoprecipitates from extracts of cells infected singly with either AcUL8 (lane 4) or AcUL30 (lane 5).

ELISAs to measure the POL/UL8 interaction. To investigate the interaction between POL and UL8 in greater detail, two separate ELISAs were developed. In one, POL was coated onto microtiter wells and binding of added UL8 was monitored with a UL8-specific MAb, while in the other assay, UL8 was coated onto microtiter wells and binding of added POL was monitored with a POL-specific MAb. The purified proteins used for the assay were essentially homogeneous as judged by SDS-PAGE analysis and coomassie blue staining: representative preparations have been shown in earlier publications (Marsden *et al.*, 1994; Parry *et al.*, 1993). In the first assay, binding of UL8 protein to POL-coated microtiter wells was detected with either MAb804 or MAb805 and the absorbance was dependent on the presence of the antibody (data not shown). Figure 2A shows the characteristics of the assay and demonstrates that the amount of either MAb bound was dependent on the presence of both POL and UL8 proteins. An amount of 0.04µg of POL was sufficient to give a good signal in this assay and was used throughout. At amounts of UL8 above 0.2µg/well some absorbance was detected in the absence of POL. Therefore, 0.2µg of UL8 protein per well was used in all subsequent experiments which produced an absorbance that corresponded to nearly the top of the steep initial rise.

In the second assay, the most sensitive of the POL-specific antibodies (Marsden *et al.*, 1996) that were tested for detection of POL-binding to UL8-coated wells was MAb 13185 (Figure 2B) and this MAb was used throughout subsequent experiments. Other POL-specific antibodies, eg. MAb 13088 and MAb 132129, gave about half the signal, while MAb 13429 and a control MAb, RWP3, gave no signal (data not shown). Again the

1 signal was dependent on the presence of both POL and
2 UL8 proteins (Figure 2B) and POL-specific antibody
3 (data not shown). Preparations of purified POL and UL8
4 protein were again titrated to determine the optimum
5 amounts to be used in this assay. It was found that
6 0.2 to 0.4 $\mu\text{g}/\text{well}$ of UL8 and 0.15 to 0.2 μg of added
7 POL gave a good signal corresponding to nearly the top
8 of the steep initial rise. These two ELISAs thus
9 provide fast and convenient assays for monitoring the
10 interaction between the two proteins.

11
12 *Specific inhibition of the POL/UL8 interaction by*
13 *UL8-reactive MABs.* Since it was possible that some of
14 the MABs reactive against UL8 might bind the molecule
15 close to the site of interaction with POL, the
16 UL8-specific MABs were screened for ability to inhibit
17 UL8 binding to POL-coated microtiter wells. Two MABs,
18 RwP3 and 105gD, that did not react with UL8 protein
19 were used as controls. Doubling dilutions of each
20 ascitic fluid were made, starting with an 8-fold
21 dilution, and were mixed with an equal volume of UL8
22 protein to give a final concentration of 0.2 μg of UL8
23 protein per 50 μl . After incubation for 1 h at 37°C,
24 the mixture was added to POL-coated microtiter plates
25 and the assay was processed in the usual manner. The
26 average absorbance in 14 wells in the absence of any
27 MAB was 0.945 (standard deviation = 0.122) and all
28 absorbance readings were normalized to this value so
29 that a relative absorbance of 1 corresponds to an
30 absorbance of 0.945. The results for 13 of the
31 UL8-specific MABs are presented in Figure 3 in which
32 the relative absorbance values for each of the 8
33 concentrations tested for each MAB are presented as
34 bars: the filled bar represents the 8-fold dilution
35 and subsequent doubling dilutions are represented by
36 progressively less densely shaded bars until the

1 1024-fold dilution open bar. Three patterns or
2 reactivity were observed. The top panel contains those
3 antibodies that did not reduce the relative absorbance
4 below 0.50 and which were classified as non-inhibitory.
5 The middle panel contains those antibodies that reduced
6 the relative absorbance to less than 0.25 and were
7 classified as inhibitory. The bottom panel contains
8 antibodies that reduced the absorbance to between 0.25
9 and 0.50. This latter group of antibodies was not
10 classified. Each antibody was tested between 2 and 4
11 times and the results from experiment to experiment
12 were in good agreement. The behaviour all 16 MAbs is
13 summarized in Table 1, which list the 5 consistently
14 inhibitory antibodies. The epitopes recognized by
15 those antibodies that inhibit the POL/UL8 interaction
16 are likely to lie at or near residues on UL8 involved
17 in its interaction with POL.

18
19 *Mapping of the epitopes recognized by the UL8-specific*
20 *MAbs.* Seven of the 8 MAbs which detected insect
21 cell-expressed UL8 in a Western blot were also
22 sufficiently sensitive to allow detection of UL8
23 expressed in BHK cells transfected with plasmid pE8
24 (MAbs 809, 811, 812, 814, 817, 818 and 819). In order
25 to determine approximate locations for the epitopes
26 recognised, we tested their ability to detect a series
27 of N- and C-terminally truncated UL8 molecules
28 expressed from derivatives of pE8. Western blots, each
29 containing an identical array of extracts from BHK
30 cells transfected with mutated plasmids, were reacted
31 with individual MAbs or with a polyclonal anti-UL8
32 antiserum (094) and representative results are shown in
33 Fig. 4. The polyclonal serum efficiently detected each
34 of the UL8 products (panel A). Three distinctive
35 patterns of reactivity were observed with the MAbs
36 indicating that the epitopes mapped to three distinct

regions. MAbs 811 (B) and 812 (not shown) reacted with all the truncated proteins indicating that they recognized an epitope lying in the region of amino acids 165 - 253 (designated as region 1). MAbs 809 (C) and 814 (not shown) reacted with all the N-terminally truncated proteins and with products lacking up to 79 but not 280 amino acids from their C-terminus indicating the presence of an epitope between amino acids 470 and 671 (region 2). Further analysis revealed that both these MAbs were able to detect a deleted form of UL8 lacking 196 amino acids from its C-terminus thereby narrowing down the region containing the epitope(s) to amino acids 470-554 (data not shown). It is not yet known whether the MAbs recognized the same or distinct epitopes within regions 1 and 2.

MAbs 817 (D), 818 and 819 (not shown) failed to react with truncated proteins lacking 33 or more amino acids from their C-termini suggesting that they recognize one or more epitopes close to the C-terminus of UL8 (amino acids 717 - 750, region 3). To determine whether amino acids from this region were sufficient for recognition and to define the epitope(s) more closely series of 8 overlapping peptides that spanned and extended 3 amino acids upstream of region 3 were synthesized (Table 2) and tested for reactivity by ELISA with the MAbs. Fig. 5 shows that the three MAbs behaved identically and reacted predominantly with the C-terminal 29 amino acids of UL8 contained in peptide 5 and to a lesser extent with the slightly shorter peptides 3 and 4. Removal of 4 amino acids from the N-terminus or 12 from the C-terminus of peptide 5 reduced the signal to background levels. It is therefore probable that all three MAbs recognize the same epitope located within the C-terminal 29 amino acids of UL8 and minimally involving the region spanning amino acids 727-739.

1 *Inhibition of the POL/UL8 interaction by UL8 peptides.*

2 The finding that all three of the MABs (817, 818 and
3 819) that mapped within the C-terminal 29 amino acids
4 of UL8 inhibited the interaction between UL8 and POL
5 prompted us to examine the role of the C-terminal amino
6 acids of UL8 in binding. Peptides 1 - 8 (Table 2) were
7 tested for their ability to block the interaction of
8 UL8 with POL. Peptides were dissolved in 100mM Tris-
9 HCl (pH 8.0) plus 0.1% Tween 20 at different
10 concentrations and incubated with POL for 15min to
11 allow the peptides to bind to POL. The mixtures were
12 then added to microtiter wells coated with UL8 and the
13 amount of POL bound was determined after 1h. Figure 6
14 shows the results for peptides 5 and 7 and a control
15 peptide, RT85. Peptides 5 and 7, which could not be
16 analysed at concentrations higher than those shown
17 because of their limited solubility, were markedly
18 inhibitory. The control peptide was non-inhibitory,
19 even at 500 μ M. The concentration of each peptide
20 required to reduce POL binding by 50% (the IC₅₀ value)
21 was determined in at least 3, and on average 5,
22 independent experiments. The averages of these values
23 are listed in Table 2 together with the standard
24 deviations for peptides 5 and 7. The different IC₅₀
25 values, ranging from 2.25 μ M to non-inhibitory, suggest
26 that the observed inhibition is peptide-specific.

27
28 To obtain additional evidence for sequence specificity
29 of the most inhibitory peptide, an additional peptide
30 was made that contained the same amino acids as peptide
31 7 but in jumbled order, and was tested for inhibition
32 of the POL/UL8 interaction. The jumbled peptide, 7J,
33 was made by linking residues from alternately the N-
34 and C- termini of peptide 7. Thus, if the order of the
35 20 residues in peptide 7 is represented as 1, 2, 3,
36 ...18, 19, 20, that in 7J was 1, 20, 2, 19, ...12, 10,

11. In the experiment shown in Figure 7, the IC_{50} value for peptide 7 was approximately $1\mu M$ while peptide 7J was 20-fold less active with an the IC_{50} value $>20\mu M$.

Inhibition of the POL/UL8 interaction does not require prior incubation of peptide 7 with POL. In the previous experiment, the peptides had been pre-incubated with POL to increase the likelihood that they would inhibit its interaction with UL8 by allowing prior formation of a peptide-POL complex. We next investigated whether the preincubation step was necessary. The data presented in Figure 8 show that the IC_{50} values (approximately $1\mu M$) for the two curves are indistinguishable, demonstrating that inhibition does not require prior incubation with the peptide and suggesting that the POL/UL8 interaction might be a weak one.

EXAMPLE 2 : Isolation and characterization of POL-specific Monoclonal antibodies

MATERIALS AND METHODS

Cells. P3-X67-Ag8 myeloma (P3) cells were grown in Dulbecco's MEM with 10% foetal calf serum, 10% horse serum, 8mM glutamine and gentamicin. *Spodoptera frugiperda* (Sf) cells were grown at 28°C in TC100 medium with 5% heat-inactivated foetal calf serum, antibiotics and neomycin. All reagents were supplied by Gibco/BRL and used as recommended by the suppliers except where noted.

Production and purification of proteins. POL was extracted (Gottlieb et al., 1990) from Sf cells infected with recombinant baculoviruses AcUL30 (Stow, 1992). POL protein was purified by a modification

1 (Marsden *et al.*, 1994) of the procedure described by
2 Gottlieb *et al.* (1990).

3

4 DNA fragments encoding portions of POL were subcloned
5 from plasmid pE30 which encodes the full length protein
6 (Stow *et al.*, 1993). Convenient restriction
7 endonuclease fragments of the UL30 gene were purified
8 and inserted in-frame into the appropriate vector from
9 the pQE30, pQE31, pQE32 series (Qiagen). The resulting
10 plasmids specify fusion proteins with an N-terminal
11 extension of approximately 25 α , including a stretch
12 of 6 histidine residues. *E.coli* strain XL-1 blue cells
13 (Stratagene) transformed with the following plasmids
14 were used:- pPQ223 encoding α 1-212 (fragment 1),
15 pPQ101 encoding α 162-316 (fragment 2), pPQ3 encoding
16 α 308-658 (fragment 3), pPQ117 encoding α 597-975
17 (fragment 4), pPQ24 encoding α 875-1119 (fragment 5),
18 pPQ136 encoding α 1072-1145 (fragment 6) and pPQ131
19 encoding α 1128-1235 (fragment 7). XL-1 blue cells
20 transformed with the vector pQE32 served as a control.
21 Synthesis of the UL30 fragments was induced following
22 treatment of *E.coli* cultures with IPTG (optimum
23 conditions were 0.1 - 1.0 mM IPTG for 1 - 5 hours
24 depending on the construct).

25

26 *Preparation of antibodies.* Donor mice for MAb
27 production received 3 subcutaneous injections at weekly
28 intervals, the first with complete Freund's adjuvant
29 (CFA) and the next two with incomplete Freund's
30 adjuvant (IFA). Three to five weeks later the mice
31 were boosted with antigen in PBS intraperitoneally and
32 test bled. Spleen donors received a further boost with
33 antigen intraperitoneally four days before the fusion.
34 Mouse spleen cells were fused to SP3 cells in the UL30
35 fusion using polyethylene glycol 1000. Fused cells
36 were plated at 3×10^5 cells/well in selective medium

1 containing HAT. Purified UL30 protein was used both
2 for immunising donor mice and for assaying secreted
3 antibodies by ELISA. The amount of protein for each of
4 the 3 initial immunisations was 10 µg for POL, while
5 for the boosts 20 µg of POL were used. ELISA assays
6 for mouse antibodies were performed with the spent
7 medium of growing hybridoma cells or with
8 immunoglobulin (Ig) purified from it. Purification was
9 achieved by precipitation of the Ig with ammonium
10 sulphate at 50% saturation or by a more rigorous
11 procedure whereby the medium was dilapidated with
12 Cab-o-sil (BDH) followed by ammonium sulphate
13 precipitation. Finally, the precipitate was dissolved
14 in and dialysed against 20mM Na-phosphate buffer pH7.0,
15 and further purified using a protein G suberose column
16 (Pharmacia): bound Ig was eluted with 0.1M glycine
17 pH2.7, neutralised, aliquoted and stored frozen.

18
19 Polyclonal antisera 514 specific for POL was raised in
20 rabbits by 5 intramuscular injections at fortnightly
21 intervals, each of 5µg of purified POL. The first
22 immunisation was in CFA while subsequent immunisations
23 were in IFA.

24
25 *ELISA.* Plates were coated at 37°C overnight with
26 0.25µg per well of purified UL30 protein: an amount
27 chosen by initial checkerboard titrations. Tissue
28 culture supernatants were added for 1 hour at ambient
29 temperature. Bound antibodies were detected with horse
30 radish peroxidase (HRP) coupled to anti-mouse Ig
31 (Scottish Antibody Production Unit), and the
32 chromogenic substrate ABTS (Sigma).

33
34 *Western blots.* Cells were solubilised with denaturing
35 sample buffer and proteins were separated by 17.5% SDS
36 polyacrylamide gel electrophoresis (SDS-PAGE) and

1 transferred to nitrocellulose membranes. The membranes
2 were incubated with purified Ig, and bound Ig was
3 visualised using HRP coupled to anti-mouse Ig for Mabs
4 or protein A for rabbit antibodies (both Sigma), and
5 chromogenic substrate 4-chloro-1-naphthol (Bio-Rad).
6 Molecular weights were estimated by comparison with
7 standard markers (Amersham 46K-2.35K).
8

9 *Immunoprecipitation.* Sf cells were infected with
10 recombinant virus AcUL30 (Stow, 1992) or parental virus
11 AcRP23lacZ (Possie & Howard, 1987), and incubated at
12 28°C overnight. Infected cells were labelled with
13 100uCi/ml of [³⁵S] methionine (Amersham) from 24 to 31
14 hours post-infection (pi). They were then washed and
15 solubilised in extraction buffer (0.5% Nonidet P40,
16 0.5% Na deoxycholate, 10% glycerol, 0.1M Tris HCl, pH8)
17 for 1 hour on ice. 50µl hybridoma supernatant was
18 incubated overnight at 4°C with 20µl of ³⁵S-labelled
19 extract and 5µl sheep anti-mouse Ig. Complexes were
20 precipitated with protein A Sepharose, eluted by
21 boiling with elution buffer (2% SDS, 5%
22 2-mercaptoethanol, 20% glycerol, 0.125M tris HCl,
23 pH6.8, bromphenol blue) and separated by 5-12.5%
24 gradient SDS-PAGE.
25

26 *Immunofluorescence.* BHK cells grown on coverslips
27 were infected with approximately 5 plaque forming units
28 wt HSV-1 per cell. At 5 hour pi cells were fixed in 2%
29 paraformaldehyde and permeabilised with 0.5% Nonidet
30 P40. Coverslips were incubated first with the
31 monoclonal antibody (undiluted supernatant) and then
32 incubated with anti-mouse Ig conjugated to fluorescein
33 isothiocyanate and examined under a Nikon microphot-SA
34 microscope.
35

1 RESULTS

2
3 *Isolation and characterisation of POL-specific MABs.*

4
5 Fourteen hybridoma lines were developed that secreted
6 antibodies which bound specifically to purified POL
7 coated onto microtiter plates. These antibodies were
8 tested for reactivity in immunoprecipitation,
9 immunofluorescence and western blotting assays and the
10 findings are summarised in Table 3. Eight of the 14
11 MABs were positive in immunoprecipitation assays and
12 reacted with a single major protein of the size
13 expected for intact POL (data not shown). Of the four
14 MABs that were positive in immunofluorescence assays,
15 the most strongly reactive was 13429. Eight MABs were
16 positive by western blotting. Three MABs, (13185,
17 13429 and 13628) were reactive in all of the
18 immunological assays.

19
20 The epitopes on POL recognised by those MABs that
21 reacted on western blots, were mapped using a series of
22 seven fragments of gene UL30 that spanned the entire
23 open reading frame. The fragments, designated 1 to 7,
24 contained POL residues 1-212, 162-316, 308-658,
25 597-975, 875-1119, 1072-1145 and 1128-1235 and have
26 expected molecular masses of 23200, 17822, 38858,
27 41863, 27030, 7718 and 11581 respectively. With the
28 exception of fragment 1 with which no reactivity was
29 observed, the POL-specific polyclonal antiserum 514
30 reacted with a polypeptide compatible (within the
31 limits of SDS-PAGE) with the expected size of each of
32 the fragments (Figure 11) demonstrating the presence of
33 fragments 2-7 in the extracts. The faster migrating
34 bands reactive with antiserum 514 are probably
35 proteolytic breakdown products of the fragments.
36 Panels B, C and D show the specific recognition of

1 fragments 3, 2 and 5 by MAb 13088, MAb 13129 and
2 MAb 13185 respectively. The fragments recognised by
3 the other western-blot reactive MAbs were determined in
4 the same manner (data not shown) and all are listed in
5 Table 3, together with the deduced approximate location
6 of the epitope.

7 8 DISCUSSION

9
10 To our knowledge, this is the first isolation and
11 characterisation of monoclonal antibodies specific for
12 the POL protein of HSV. Within the fourteen MAbs
13 specific for the catalytic subunit of the DNA
14 polymerase, eight distinct specificities can be
15 recognised. This can be deduced from the data in
16 Table 3, which shows that six distinct patterns of
17 reactivities (A-F) in immunofluorescence (IF),
18 immunoprecipitation (IP) and western blotting (WB)
19 assays were observed. Pattern A, represented by MAbs
20 13185, 13429 and 13628, is formed by those MAbs that
21 are positive in all three assays. It contains two
22 specificities: MAbs 13185 and 13429 recognise an
23 epitope between α 976-1071 whereas the epitope
24 recognised by MAb 13628 lies between α 1120-1127.
25 Similarly, MAb 13129, 13488 and 13528, comprising
26 pattern B, define two epitopes.

27
28 None of the POL-specific antibodies inhibited DNA
29 polymerase activity. The nine different epitopes
30 recognised by the antibodies are widely spread over POL
31 though none is located near its C-terminus. These
32 findings are consistent with our earlier observation
33 that the C-terminal 27 α of POL are responsible for at
34 least 75% of the binding energy of POL to UL42 protein.
35 Interestingly, of the panel of 13 UL42-specific MAbs
36 that was recently described by Scheaffer et al. (1995),

all but one of the epitopes were outside the minimal active portion of the protein and none interfered with the POL/UL42 interaction.

EXAMPLE 3 : Demonstration of an interaction between HCMV UL54 and UL102 proteins and inhibition of that interaction by UL102 C-terminal and C-proximal peptides

METHODS

Expression of the HCMV UL102 and UL54 gene products.

Recombinant baculoviruses expressing HCMV genes UL102 and UL54 under the control of the polyhedrin promoter were generated as described below.

A 3.7 kb fragment spanning nucleotides 76904-80636 of HCMV DNA (Chee et al., 1994) and containing the HCMV UL54 ORF was amplified from a cloned copy of the HindIII F fragment of HCMV strain AD169 by PCR. The primers used were:
5'-ATTATCTAGACCGCTATGTTTTTCAACCCG-3' and
5'-TATATCTAGACATCATCACCGTCCCCAGTCA-3' which contained XbaI sites (underlined). The PCR-generated fragment was cleaved with XbaI and initially cloned into the XbaI site of pUC19. The XbaI fragment was then recloned into the XbaI site of the baculovirus transfer vector pACYMX1 (Stow, 1992) downstream of the polyhedrin promoter to generate plasmid PY54. The entire XbaI fragment was sequenced to confirm the presence of the authentic UL54 gene.

A 2.7 kb fragment spanning nucleotides 146510-149208 of HCMV DNA (Chee et al., 1994) and containing the HCMV UL102 ORF was amplified from a cloned copy of the HindIII R fragment of HCMV strain AD169 by PCR. The primers used were 5'-ATTAGGATCCTTCTGTCCGAGGATGACCGCT-3'

1 and 5'-ATTAGGATCCACGTCACACGCTAAGAGC-3' which contained
2 BamHI sites (underlined). The PCR-generated fragment
3 was cleaved with BamHI and cloned firstly into the
4 pUC19 BamHI site. The UL102-containing BamHI fragment
5 was then inserted into the BamHI site of transfer
6 vector pAcYM1 (Matsuura et al., 1987) to generate
7 plasmid PY102. The presence of the authentic UL102
8 gene was confirmed by DNA sequencing of the entire
9 BamHI fragment.

10
11 The transfer plasmids (PY54 and PY102) were separately
12 cotransfected with Bsu36I-cleaved DNA of the parental
13 baculovirus AcPAK6 (Bishop, 1992) into *Spodoptera*
14 *frugiperda* (Sf) cells and recombinant baculoviruses
15 were isolated as described by Kitts et al. (1990). The
16 presence of the desired genes was confirmed by Southern
17 blot analysis using the amplified fragments as probes.
18 Resulting viruses AcUL54 and AcUL102 contain the UL54
19 and UL102 genes, and stocks were prepared and titrated
20 as described (Brown and Faulkner, 1977; Matsuura et
21 al., 1987).

22
23 *Purification of UL54 and UL102.* Proteins UL54 and
24 UL102 were extracted from Sf cells infected with
25 recombinant baculoviruses AcUL54 and AcUL102 and
26 purified as was described for the HSV-1 homologues UL30
27 and UL8 respectively (see POL/US interaction assays,
28 Example 1).

29
30 *Measurement of DNA polymerase activity.* Activity was
31 measured by incorporation of [³H]dTTP into a
32 poly(dA)-oligo(dT)₁₂₋₁₈ template using a concentration of
33 50 mM KCl, previously found to be optimal for the HCMV
34 enzyme (Ertl et al., 1991). The reaction mixture
35 (final vol. 100 µl) contained 75 mM Tris HCl pH 8.0,
36 1.67 mM 2-mercaptoethanol, 6.5 mM MgCl₂, 1 µg poly

(dA)-oligo (dT), 50 mM KCl, 40 µg BSA and 10 ng UL54 protein (HCMV DNA polymerase). Reactants were mixed on ice and the reaction was initiated by addition of 1.7 µM ³H-dTTP (specific activity 3.75 Ci/mmol) and transfer to 37°C. Samples of 10 µl were taken 5, 10, 15 and 20 minutes later, and spotted onto Whatman DE81 ion exchange discs which had been soaked in 0.1M EDTA and air dried. The discs were given three 10 minute washes with 5% Na₂HPO₄, two 5 minute washes with water and two 30 second washes with methylated spirits. They were air dried and counted in a scintillation counter with 5 ml of Ecoscint A (National Diagnostics, Kimberley Research).

Oligopeptides. Peptides were synthesized by continuous flow Fmoc chemistry as previously described (Atherton and Sheppard, 1989; McLean et al., 1991). The peptides listed in Table 4 were purified by preparative reverse-phase HPLC. The relative molecular masses of the purified peptides was determined by matrix-assisted, laser desorption time-of-flight mass spectrometry and corresponded to the desired products.

Antibodies. The hybridoma cell line that secretes monoclonal antibody (MAb) 13815 has been deposited with the European Collection of Cell Cultures (reference number 96072640). Antiserum 113, specific for HSV-1 UL30 (POL), was raised against a peptide corresponding to the C-terminal 15 amino acids of the protein and has been described previously (Marsden et al., 1994). Antiserum 144, specific for HCMV UL54 protein, was raised in rabbits against peptide HLEPAFLPYSVKAHE that corresponds to the C-terminal 15 amino acids (residues 1226-1240) of UL54. Antiserum 373, specific for HCMV UL102 protein, was raised in rabbits against peptide VLSSALPSVTSSSSG that corresponds to residues

809-823 of the 873 residue UL102. The peptides were made as multiply antigenic peptides (Tam, 1988) of general structure (peptide sequence)₄K₃A as such peptides have been shown to generate sera with higher anti-protein titers (McLean et al., 1991).

UL54/UL102 interaction assays. ELISA assays, similar to those described for HSV-1 POL/UL8 (see Example 1 above) were established with purified HCMV UL54 and UL102 proteins. Both proteins were diluted in PBS to the required concentrations. For the assay, microtiter wells were coated overnight with purified UL102, at the concentrations indicated in the text, and blocked with 100 μ l of 2% BSA in PBS for 1 h at 37°C. After blocking, the plates were washed extensively with PBS containing 0.3% Tween 20 and blotted dry. Then 50 μ l of purified UL54, at the concentrations indicated in the text, were added to each well and incubated for 1 h at 37°C. Following further washes, 50 μ l of UL54-specific antiserum, diluted in PBS containing 2% FCS was reacted for 1 h at 37°C. The wells were again extensively washed and bound antibody was detected with 50 μ l/well of HRP-conjugated protein A (Sigma) diluted 1:500 in PBS containing 2% FCS. After further washes, chromogenic substrate ABTS was added. Peptides, diluted in 100mM Tris-HCl (pH 8.0) plus 0.1% Tween 20, were added to the interaction assay as described in the text.

RESULTS

Nucleotide sequence of HCMV gene UL102. Our independently determined sequence of the entire cloned fragment that spanned nucleotides 146510-149208 (data not shown) was the same as that originally reported in the sequence of the entire genome of HCMV strain AD169

(Chee et al., 1994) with the exception of nucleotide 146753. In agreement with Smith and Pari (1995), we found that this residue is a cytosine rather than a guanosine that changes the putative in-frame stop codon TAG at position 146751 to the tryptophan codon TAC. We therefore concur with the interpretation of Smith and Pari (1995), that the first in-frame stop codon is at nucleotide 149105 and that gene UL102 has the capacity to encode a protein of 873 amino acids with a molecular mass of approximately 100K.

Purification of proteins. HCMV UL102 and UL54 proteins were extracted in the same buffers, and purified by the same procedures previously used for the homologous HSV-1 UL8 and UL30 proteins (see above). Purification was monitored using the UL102- and UL54-specific antisera 373 and 144 respectively. Figure 12 shows a Commassie blue stained gel of purified UL102 (lane 2) and UL54 (lane 3). The marker proteins show that the proteins migrate to positions compatible with their predicted sizes.

Additional evidence for the authenticity of the UL102 protein was provided by the specific reaction of immune serum 373, but not the pre-immune serum, with the protein. Figure 13 shows a western blot of two different extracts from AcUL102-infected Sf cells (EXT-1 and EXT-2) together with peak UL102-containing fractions from the DEAE-sepharose column (DEAE-2) and the hydroxylapatite column (HA-2). The purification procedure removes a number of faster migrating UL102-related protein bands. It is noteworthy that the pre-immune serum does not react with any bands in these same fractions (Lanes 6 - 9). Alignment of the blot with the Commassie blue stained gel showed that the band that reacted with antibody 144 comigrated with the

1 purified protein and migrated to the same position with
2 respect to the protein markers (alignment not shown).

3
4 Additional evidence for the authenticity of the UL54
5 protein was provided by the specific reaction of immune
6 serum 144, but not the pre-immune serum, with the
7 protein (Figure 14). Furthermore, the purified protein
8 was able to catalyse incorporation of [³H]dTTP into an
9 poly(dA)-oligo(dT)₁₂₋₁₈ template as would be expected of
10 the catalytic subunit of the HCMV DNA polymerase
11 (Figure 15).

12
13 *Development of an ELISA to measure the UL54/UL102*

14 *interaction.* To provide evidence that the
15 UL54-specific antiserum 144, directed against the
16 C-terminal 15 amino acids of the protein, might be
17 suitable for measuring the UL54/UL102 interaction, we
18 tested whether antiserum 113, directed against the
19 C-terminal 15 amino acids of HSV-1 UL30 (POL) could be
20 used to detect the HSV-1 UL30/UL8 interaction. As a
21 control we used the UL30-specific MAb 13185 that had
22 previously been used to monitor the HSV-1 interaction.
23 The assay was performed as described previously.
24 Briefly, plates were coated with UL8 and bound UL30 was
25 detected with MAb 13185 followed by HRP-conjugated anti
26 mouse IgG and chromogenic substrate. The absorbance
27 was recorded at 405nm. The results are shown in Figure
28 16 and are presented in groups of 4 bars. Bar 1 shows
29 absorbance in wells with both UL8 and UL30, bars 2, 3
30 and 4 show the absorbance when UL30, UL8 or both UL8
31 and UL30 respectively were omitted. Data in groups A
32 and B show the results with and without MAb 13815, and
33 confirm our previous findings showing that the signal
34 is dependent on the presence of HSV-1 UL8, UL30 and MAb
35 13185. Data in groups C, D, E and F show similar
36 experiments in which the detecting antibody was rabbit

1 serum 113 diluted 10^3 -, 10^4 - or 10^5 -fold or omitted (No
2 RAb). Bound antibody was detected with HRP-conjugated
3 protein A. The data show that in this assay, the
4 signal is dependent on the presence of HSV-1 UL8, UL30
5 and the C-terminal antiserum 113. Thus, this
6 C-terminal antiserum can be used to monitor the HSV-1
7 UL30/UL8 interaction.

8

9 These findings suggested that the rabbit serum 144,
10 specific for the C-terminus of HCMV UL54 might enable
11 the HCMV UL54/UL102 interaction to be monitored. The
12 data presented in Figure 14 indicated that this serum
13 could be diluted 5-fold and give an acceptable signal
14 with UL54 bound directly to wells, and the antibody was
15 accordingly used at that concentration. For the
16 interaction assay, wells were coated with amounts of
17 UL102 ranging from zero to 0.4 μ g per well. UL54 was
18 added in amounts ranging from ranging from zero to 0.6
19 μ g per well. The results (Figure 17) show that the
20 signal: 1) is dependent on the presence of both
21 proteins, 2) increases as the amount of UL102 is
22 increased and 3) increases as the amount of UL54 is
23 increased, up to 0.4 μ g per well. We interpret these
24 data as evidence for an interaction between UL54 and
25 UL102.

26

27 *Inhibition of the UL54/UL102 interaction by UL102*

28 *peptides.* We wished to demonstrate that peptides at or
29 near the C-terminus of other herpesvirus homologues of
30 HSV-1 UL8 would disrupt the interaction between the
31 homologues of UL8 and POL. To do this, the peptides
32 listed in Table 4 were tested for their ability to
33 block the interaction of HCMV UL102 with HCMV UL54.
34 Peptides, diluted in 100 mM Tris-HCl (pH 8.0) plus 0.1%
35 Tween 20, together with 0.4 μ g UL54, were added to
36 microtiter wells pre-coated overnight with 0.4 μ g

1 UL102. The amount of UL54 bound was determined after
2 1h by measuring the absorbance at 405 nm as described.
3 The absorbance in the absence of any peptide, $1.061 \pm$
4 0.033 , was determined from six wells. The background
5 absorbance in the absence of any UL54, 0.240 ± 0.025 ,
6 was also determined from six wells and was subtracted
7 from all values. Figure 18 shows the results, derived
8 from the average of duplicate wells, for peptides 1 and
9 2 and a control peptide, RT85. The concentration of
10 each peptide required to reduce UL54-binding by 50%
11 (the IC_{50} value) was determined and is listed in Table
12 4.

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